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ABSTRACT: The objectives of this study were to provide data that can be used to predict exposure-based effects of TNT in aged soil on four endpoint organisms representing two trophic levels. These data can be used for defining criteria or reference values for environmental management and for conducting specific risk assessment.

Dose-response experiments formed the basis for evaluating the toxic effects and transfer of contaminants from soil into two trophic levels, taking bioavailability-modifying soil characteristics into account. Short-term exposure tests were conducted to explore the acute toxicity for the test organisms of TNT-spiked artificial soils and of the aged TNT-contaminated soil to be included in the subsequent long-term exposure tests. In these tests, plants were exposed for 10 days, and seed germination was determined. Worms were exposed for 14 days, and survival was recorded. Long-term exposure tests were conducted to evaluate chronic, sublethal toxicity and transfer of aged soil-based explosives, with TNT as the main contaminant. In these tests, plants were exposed for 55 days in the greenhouse, biomass was determined, and residues of explosives parent compounds and TNT metabolites were analyzed using HPLC techniques. Worms were exposed for 28 days (*Eisenia fetida*) and 42 days (*Enchytraeus crypticus*) in the laboratory, biomass and number were determined, and tissues were analyzed for explosives compounds.

Both plant test species, *Lolium perenne* and *Medicago sativa*, tolerated TNT concentrations up to 171 mg kg^{-1} dry weight (DW) during short-term exposure. In the longer term the plants were less tolerant of TNT, but *L. perenne* was more tolerant than *M. sativa*. An effective concentration causing a 20 percent decrease in plant biomass (EC20) of 2.4 and EC50 of 7.2 mg TNT kg^{-1} soil DW was derived for *L. perenne* from linear regression. An EC50 of $\leq$2.7 mg TNT kg^{-1} was found for *M. sativa*, based on the observation that these plants died at TNT concentrations >5.4 mg kg^{-1}. TNT metabolites (2ADNT, 4ADNT), RDX, and HMX were recovered in *L. perenne* shoots and roots. Only the TNT metabolite concentrations in shoots increased significantly with soil TNT concentration.

Among the worm test species, *E. fetida* tolerated TNT concentrations up to 100 mg kg^{-1} during short-term exposure. Fifty percent of these *E. crypticus* individuals died at a TNT concentration as low as 10 mg TNT kg^{-1}, and all died at higher concentrations.

In the longer term the worms were less tolerant of TNT, but *E. fetida* was more tolerant than *E. crypticus*. An EC20 of 1.2 and EC50 of 3.6 mg TNT kg^{-1} soil DW was derived for *E. fetida* from linear regression. An EC50 of $\leq$2.15 mg TNT kg^{-1} was found for *E. crypticus*, based on the observation that these worms died at a TNT concentration of 4.3 mg kg^{-1}. No explosives parent compounds or metabolites were recovered in the worms.

Because only the effects of soil-TNT concentration on the biomass of *L. perenne* and *E. fetida* were significant, the toxicity of the soil was attributed mainly to the contamination by TNT. However, the other explosives identified in the soil mixtures prior to the tests may have contributed also. Clay amendment did not significantly affect the plant and worm responses.

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Preface

The studies reported herein were conducted by the Environmental Laboratory (EL) of the U.S. Army Engineer Research and Development Center (ERDC), Vicksburg, MS. The research was conducted as part of the U.S. Army Corps of Engineers Environmental Quality Basic Research Program. Dr. M. John Cullinane, EL, was the Program Manager.

Personnel who cooperated in the execution of the study and preparation of this report included Dr. Elly P.H. Best, Dr. Henry E. Tatem, Ms. Melissa L. Wells, Environmental Risk Assessment Branch (ERAB), Environmental Processes and Engineering Division (EPED), EL; and Ms. Kaaren N. Geter and Mr. Bryan K. Lane, Analytical Services, Incorporated, Vicksburg, MS. Technical reviews were provided by Drs. Judith C. Pennington, Laura S. Inouye, and Joan Clarke, EPED. The help of Ms. Margaret Richmond with the explosives analyses is gratefully acknowledged.

The study was conducted under the direct supervision of Dr. Lance D. Hansen, Chief, ERAB, and the general supervision of Dr. Richard E. Price, Chief, EPED, and Dr. Edwin A. Theriot.

COL James R. Rowan is Commander and Executive Director of ERDC. Dr. James R. Houston is Director.

1 Introduction

Source and Distribution of Explosives in Soils

Explosives, including 2,4,6-trinitroluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and associated byproducts and degradation compounds, have been released into the environment from munitions production and processing facilities. TNT has been identified at 19 National Priority List for Superfund Cleanup (Fed. Reg. 60: 20330) sites across the U.S. (ATSDR 1995). These sites include U.S. Army Ammunition Plants (AAPs) and load, assemble, and pack (LAP) processing sites. TNT is also present in the environment as a result of decommissioning activities and through field usage and disposal activities such as open burning. TNT has been found in environmental media only in the vicinity of such sites.

Explosives-contaminated soil concentrations are extremely heterogeneous, ranging from 0.08 to 87,000 mg kg^{-1} for TNT, from 0.7 to 74,000 mg kg^{-1} for RDX, and from 0.7 to 5,700 mg kg^{-1} DW for HMX and can vary from site to site (Talmage et al. 1999).

Evaluation of Potential Toxicity of Explosives-Contaminated Soils; Screening Benchmarks

The use of toxicity data is an important tool in predicting the effects of contaminants on populations, defining criteria or reference values for environmental management, and conducting site-specific risk assessment (Renoux et al. 2001; Robidoux et al. 2002a). To determine if concentrations at a site might be harmful to the indigenous species, the maximum measured media-specific concentration can be compared with a criterion, or screening benchmark. The criterion, or benchmark, is a concentration that should not result in adverse ecological effects to the populations of indigenous species. Both terrestrial plants and invertebrates are important, because they contribute to the functional aspects of the soil and because they play key roles in the food chain. For plants and soil invertebrates, lowest- or no-observed effect concentrations (LOECs or NOECs) have to be determined as a basis for these screening benchmarks. In plants, effective concentrations causing a 20 percent decrease in biomass, EC20s, are

often used as a measure for an LOEC. In animals, concentrations causing a 50 percent mortality, LC50s, have traditionally been used as a measure for toxicity. To date, effects-based ecotoxicological criteria for explosives-contaminated soil are extremely scarce. These would be required for safe management of the future use of decommissioned military training sites. Moreover, among the existing test data relating to TNT, concentration-dependent effects in hydroponically grown plants do not compare well with effects in plants grown in soil because the characteristics of the matrices differ. Also, effects on plants and worms of TNT spiked onto soil, and of TNT from aged soil, are difficult to compare.

Spiking can cause solvent effects. The time allowed for evaporation of the solvent prior to incubation can permit a decrease in the concentration of the parent compound and the formation of undefined degradation compounds in the test.

Aged soil may contain other contaminants, such as nitroaromatics and metals, that may confound the TNT effects. Nevertheless, below we give a brief overview of the most significant effects noted in plants and worms.

The recently published screening benchmark for TNT in soil for terrestrial plants is 30 mg kg^{-1} (Talmage et al. 1999). This value is based on the LOEC of 30 mg TNT kg^{-1} for aged soil, with a NOEC of 10 mg TNT kg^{-1} in bush bean (*Phaseolus vulgaris*) (Cataldo et al. 1989).

The recently published screening benchmarks for TNT in soil for soil invertebrates are 140 mg kg^{-1} for earthworms and 200 mg kg^{-1} for other invertebrates (Talmage et al. 1999). The benchmark for earthworms is based on the LOEC of 140 mg TNT kg^{-1} for spiked artificial soil, with a NOEC of 110 mg TNT kg^{-1} (Phillips et al. 1993). The benchmark for soil nematodes and arthropods is based on a 7-d LOEC of 200 mg TNT kg^{-1} for spiked forest soil, with a NOEC of 100 mg TNT kg^{-1} (Parmelee et al. 1993).

Effects of TNT on Plants

Standardized toxicity assays (OECD 1984; ISO 1993, 1995; USEPA 1996, 1999) are used to assess the effects of pure and mixed contaminants on terrestrial plants using such endpoints as seed germination, growth, or root elongation. The number of studies describing the phytotoxicity of explosives, such as TNT, RDX, and HMX, on higher plants is limited (for review, see Talmage et al. 1999; Sunahara et al. 2001).

TNT is taken up and metabolized by plants to 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-4,6-dinitrotoluene (4ADNT) (Palazzo and Leggett 1986; Harvey et al. 1990; Sens et al. 1998, 1999), 2,4-dinitrotoluene (2,4DNT), and 2,6-dinitrotoluene (2,6DNT) (Sens et al. 1998, 1999; Best et al. 1999). The relative order of metabolite concentration in plants is roots>stems>leaves≥seeds. The explosives residues usually identified in TNT-exposed plants are 2ADNT and 4ADNT. TNT residues have been recovered from shoots and below-ground

organs exposed to concentrated aqueous TNT solutions (20 mg L^{-1}; Palazzo and Leggett 1986) and from roots exposed to extremely high soil-TNT concentrations (471-1920 mg kg^{-1} soil DW) (Thorne 1999).

Toussaint et al. (1995) reported an EC50 of 10 μM for the effect of TNT on root elongation in lettuce (*Lactuca sativa*). Thompson et al. (1998) reported a LOEC of 22 μM for the effect of TNT on transpiration and biomass of hybrid poplar (*Populus* sp. *deltoides* × *nigra*, DN34) cuttings.

The toxicity of TNT, spiked onto soil in concentrations ranging from 25 to 1,600 mg kg^{-1}, was higher for two dicotyledonous species (cress, *Lepidium sativum* L.; and turnip, *Brassica rapa* Metzg.) than for two monocotyledonous species (oat, *Avena sativa* L.; and wheat, *Triticum aestivum* L.) (Gong et al. 1999). The LOEC was 50 mg kg^{-1} soil (cress, turnip). Oat tolerated up to 1,600 mg TNT kg^{-1} soil.

The toxicity of aged explosives from soil, with TNT as the main contaminant and TNB and metals also present, was determined in cucumber (*Cucumis sativus*) and radish (*Raphanus sativus*) during a field study (Simini et al. 1995). In this case, most of the soil toxicity was accounted for by TNT and TNB. The LOEC of TNT ranged from 7 to 19 mg kg^{-1} soil at the two locations studied. A somewhat higher LOEC, 42 mg TNT kg^{-1} soil, was found in smooth bromegrass (*Bromus inermus*) and tall fescue (*Festuca arundinacea*) by Krishnan et al. (2000). In another aged soil study (Scheidemann et al. 1998), levels above 10 mg TNT kg^{-1} were toxic and above 100 mg TNT kg^{-1} were lethal to alfalfa (*Medicago sativa*), while 500 mg TNT kg^{-1} soil still allowed biomass formation in wheat and bush bean (*Phaseolus vulgaris*).

In summary, the response of plants to TNT exposure depends on the way TNT is administered (spiked or aged), the matrix (hydroponics or soil), and the plant species. No indication of a general difference in TNT sensitivity (Tucker et al. 1989; Gong et al. 1999; Gorge et al. 1994) or TNT uptake and transformation (Scheidemann et al. 1998) between dicotyledonous and monocotyledonous plants has been identified.

Effects of TNT on Worms

Earthworms are suitable response and bioaccumulation indicators for organics as well as for metals (ASTM 1998; Kula and Larink 1998; Lokke and Van Gestel 1998). Toxicological effects in earthworms originate primarily from direct skin contact with the toxic compounds in the interstitial water. Effects of explosives on different soil invertebrate populations, including earthworms and enchytraeids, may be seen at other population levels, e.g. birds and mammals, through food chain relationships. Thus, risk assessment must take into account sublethal effects of environmental contaminants whose presence may have an impact on certain life cycle parameters of invertebrates such as worms, e.g. survival, weight change, cocoon production, and fertility.

TNT is reduced by earthworms (Renoux et al. 2000; Robidoux et al. 2002b). The only explosives residues identified were 2ADNT and 4ADNT in worms exposed to TNT-spiked soil in concentrations >50 mg kg^{-1} soil DW (Robidoux et al. 2002c).

The toxicity of spiked TNT evaluated in 14-day exposures in earthworms (*Eisenia fetida*) was lower on forest soils than on artificial soil (Phillips et al. 1993). A NOEC of 140 mg kg^{-1} was found on forest soil, and a NOEC of 110 mg kg^{-1} soil DW was found on artificial soil. A LOEC of 150 mg kg^{-1} and LC50 of 325 mg kg^{-1} were found on forest soil. The toxicity of spiked TNT for another earthworm species, *Eisenia andrei*, was lower than for *Eisenia fetida*. LOECs of 260 mg kg^{-1} on forest soil and 420 mg kg^{-1} on artificial soil were found (Robidoux et al. 1999). In the same earthworm species, *E. andrei*, TNT spiked onto forest soil greatly decreased the growth of adults at ≥136 mg kg^{-1}, and various reproduction parameters of fecundity (total and hatched numbers of cocoons) at ≥ 58.8 mg kg^{-1} (RDX at ≥46.7 mg kg^{-1} and HMX at ≥15.6 mg kg^{-1}). All these levels were higher in spiked artificial soil (Robidoux et al. 2002b).

Concentration-dependent weight loss was also reported for RDX and HMX spiked at levels of <500 mg kg^{-1} onto artificial soil, but lethality was not observed (Phillips et al. 1993).

The toxicity of aged explosives from soil, with TNT as main contaminant and metals also present, was determined in a field study (Simini et al. 1995). The LOEC of TNT ranged from 7 to 19 mg kg^{-1} soil at the two locations studied. As noted earlier, most of the soil toxicity was accounted for by TNT, although the results may have been influenced by the presence of the other contaminants, TNB, and metals.

In summary, the response of worms to TNT exposure depends on the way TNT is administered (spiked or aged), the matrix (artificial or forest soil), and the worm species. *Eisenia fetida* appears to be less sensitive to TNT than *E. andrei*. Other explosives parent compounds that often co-occur with TNT, such as RDX and HMX, are less toxic than TNT for worms.

Organisms Selected for Testing for Effects and Fate of TNT Contamination in Soil

In evaluating test species for ecotoxicity assessment, it is important to assess the ecological relevance, standardization, and sensitivity of the organisms considered. The criterion "ecological relevance" is subjective and is based on the investigator's judgment regarding a species' applicability or meaningfulness to the site being assessed. Ecological relevance can be based on the likelihood of occurrence of the test organism at the site, the functional role that the organism represents, and the relationship of the test species to the biological resource being assessed. Standardization has been defined as "the extent to which the study follows specific protocols recommended by a recognized scientific authority for conducting the method correctly" (Menzie et al. 1996). Examples of accepted

standard toxicological testing protocols are those published by the USEPA and the American Society for Testing and Materials (ASTM) or those found consistently in peer-reviewed scientific literature. In their weight-of-evidence approach, Menzie et al. (1996) rank methods that have been used in three or more peer-reviewed publications as have repute equal to that of accepted standard methods (e.g., ASTM-published methods). Sensitivity refers to the test species' proclivity of response to the physical or chemical stressor of concern.

Two plant species were selected for the tests based on their worldwide use and general acceptance in standard test procedures (ASTM 1999; USEPA 1996, 1999). The monocotyledonous *Lolium perenne* (perennial ryegrass) and dicotyledonous *Medicago sativa* (alfalfa) both have a wide geographical distribution, rapid growth, and profuse generative reproduction. In addition, their seeds germinate simultaneously within several days, and the species can be cultivated in the testing environment. Both species are relatively insensitive to organic contaminants and are widely used as a response and bioaccumulating indicator for organics contamination of soils (Page et al. 1982; Gorsuch et al. 1990; Van de Leemkuile et al. 1998; Malmberg et al. 1998). A species related to *L. perenne*, *Lolium multiflorum* (ryegrass), is widely used in Germany as a bioaccumulating indicator for organic contaminants. Both plant species are considered as moderately ecologically relevant test species, and *L. perenne* as highly standardized (Markwiese et al. 2000).

Two worm species were selected, also based on their worldwide use in standard test procedures, facilitating comparison with bioaccumulation and toxicity data of other sites, and ease of culture under laboratory conditions: the earthworm *Eisenia fetida* and the enchytraeid worm *Enchytraeus crypticus*. Both worm species are suitable bioaccumulation and response indicators for metals as well as organics and are relatively insensitive (ASTM 1998; Kula and Larink 1998; Lokke and Van Gestel 1998). *E. fetida* and *E. andrei*, the most-used laboratory test species, live preferably in organic-matter-rich soil, exhibit a litter-dwelling ecological strategy, and reproduce via cocoons. *E. crypticus* is present in the upper few centimeters of any soil at high densities. Enchytraeid worms are far smaller than earthworms and are, therefore, more frequently used for toxicological than for bioaccumulation tests. *Eisenia* species are of low ecological relevance and are highly standardized; enchytraeid worms are highly ecologically relevant and are moderately standardized test species (Markwiese et al. 2000).

Study Objectives

The objectives of this study were to provide data that can be used to predict the exposure-based effects of TNT in aged soil on four endpoint organisms representing two trophic levels. These data can be used for defining criteria or reference values for environmental management and for conducting specific risk assessments.

Dose-response experiments formed the basis for the evaluation of toxic effects and transfer of contaminants from soil into two trophic levels, taking bioavailability modifying soil characteristics into account.

2 Materials and Methods

Explosives Chemicals and Standards

Technical-grade TNT was obtained from the Central Explosives Holding Area, Waterways Experiment Station, Vicksburg, MS. The technical TNT was purified by four successive recrystallization cycles in methanol at 40 °C. Verification of the purity of TNT using HPLC analysis indicated 1 percent TNB. The purity was considered appropriate for metabolic studies. Explosives standards were purchased from Accu Standard Inc., Ellington, CT.

Plants and Worms

Seeds of *Lolium perenne* var. Linn and *Medicago sativa* var. Ladack were purchased from the Granite Seed Company, Lehi, UT.

Adult *Eisenia fetida* specimens were taken from the ERDC laboratory culture, originally purchased from Carolina Biological Supply Company, Burlington, NC. Adult *Enchytraeus crypticus* specimens were taken from an ERDC laboratory culture reared from a mass culture obtained from R. Kuperman (U.S. Army Aberdeen Proving Ground, MD) in 2001. Food for both worm species was supplied regularly as needed. It was composed of rolled oats, purchased locally, and powdered earthworm food purchased from Magic Products Inc., Amherst Junction, WI.

Range-Finding Experiment: Short-Term Exposure to TNT-Spiked, and TNT-Contaminated Aged Soil

A range-finding experiment was conducted to explore the acute toxicity for the test organisms in TNT-spiked artificial soils and in the aged TNT-contaminated soil to be included in the subsequent sublethal tests.

The following soils were used:

- Artificial soil, i.e. 70 percent (w/w) grade No 4 sand (Ash Grove, Jackson, MS), 20 percent colloidal kaolinite clay, and 10 percent 2-mm *Sphagnum* peat, prepared according to the Organization for Economic Cooperation and Development (OECD) method (OECD 1984).

- Aged, TNT-contaminated soil, containing 171 mg TNT kg^{-1} DW (see below for characteristics; Table 1).

Table 1
Characteristics of the Soils Used to Create the Soil Mixtures for the Tests and Control Soils. Mean Values and Standard Deviations (N = 5 for Explosives, N = 3 for Other Characteristics). The Levels of 2ADNT, 4ADNT, 2,4DANT, 2,6DANT, 2NT, 3NT, 4NT, 2,4DNT, and 2,6DNT Were Below Detection

Characteristic	Soil				
	NOP-LLA	NOP-Reference	Sharkey-Clay[1]	Plant Control[1]	Worm Control[1]
Explosives (mg kg^{-1} DW)					
TNT	170.85±43.20	<1.684	ND	ND	ND
TNB	23.35±5.54	<1.000	ND	ND	ND
RDX	1512.50±246.95	<3.122	ND	ND	ND
HMX	150.30±29.68	<1.913	ND	ND	ND
Nutrients (mg kg^{-1} DW)					
Nitrate-N	ND	117.93±15.71	ND	122.6±26.1	3.93
Infinite-sink P	76.2±18.8	0.98±0.24	ND	14.3±9.8	0.6±0.05
Total-K	ND	1339	ND	ND	5.3±1.2
Other					
pH_{water}	5.44±0.07	6.51±0.04	5.62±0.06	5.79	7.06±0.08
OM (% DW)	3.37±0.31	5.22±0.06	5.83±3.01	76.29	1.33±0.16
DW (% FW)	89.88±0.08	90.55±0.15	86.91±0.07	41.8	99.6±0.24
BD (g DW mL^{-1})	2.52±0.20	2.17±0.22	0.95±0.06	9.3	2.39±0.25

Note: Abbreviations: BD, bulk density; FW, fresh weight; OM, Organic matter; ND, not determined.
[1] These soils were not subjected to explosives analyses since no prior history of exposure existed.

Artificial soils were spiked with 10, 50, and 100 mg TNT kg^{-1} DW using methanol as a solvent. Non-spiked and solvent-spiked artificial soils served as controls. For plants only, an extra control potting soil was tested (Baccto R Lite potting soil, Michigan Peat Company, Houston, TX; Table 1). After spiking, the soils were mixed with a stainless steel spatula and placed in a vented fume hood without illumination for 1 h to allow the methanol to evaporate prior to exposure of the test organisms. All units were sprayed with reverse osmosis (RO) water immediately after the test organisms were placed on the soils, and, subsequently, every other day as needed.

For the plant test units, 25 seeds were placed on 5 g of soil contained in a 15-mL petri dish. Units were incubated in a walk-in growth chamber illuminated with 500–600 $\mu E\ m^{-2}\ s^{-1}$ at the seed surface at a 14-h photoperiod and

temperature of 22–26 °C. All treatments were replicated five times. The parameter for plant response was seed germination, observed as root emergence at the end of 10 days, which was long enough to observe acute toxicity.

For the worm test units, 10 specimens were tested in the following configurations: *E. fetida*, 15 g of soil contained in a 100-mL Mason jar; and *E. crypticus*, 2 g of soil contained in a 5-mL petri dish. *E. fetida* units were incubated in a walk-in growth chamber illuminated with 50 $\mu E\ m^{-2}\ s^{-1}$ at a 14-h photoperiod and temperature of 22–26 °C. *E. crypticus* units were incubated in darkness at 16 °C. All treatments were replicated five times. The parameter for worm response was survival at the end of 14 days, which was long enough to observe acute toxicity.

Toxicity and Bioaccumulation Assays: Long-Term Exposure to TNT-Contaminated Aged Soil

A sublethal, chronic toxicity test was carried out to evaluate the effects and transfer of TNT contamination from aged soil in the test organisms.

Dose-response curves for TNT concentrations between 0 and 18 mg kg^{-1} DW were constructed for both plant and animal tests. The test mixtures were prepared by mixing TNT-contaminated soil originating from the Nebraska Ordnance Plant (NOP) with clean reference soil and, in selected cases, with clay soil to modify the bioavailability of TNT (Table 1, Table 2). The test used the following treatments:

- **TNT concentration**: four, i.e., 0 (reference), 5, 10, and 18 mg TNT kg^{-1} DW. The TNT concentration range selected was based on the results of the range-finding experiment and included the range of published screening benchmarks for TNT from aged soil in plants. A concentration of 17.2 mg TNT kg^{-1} DW was the remedial cleanup goal (RG) for soil at the NOP (Price et al. 2002).

- **Clay content:** two levels, i.e., 0 and 30 percent clay (w/w).

Control potting soil served as a test to verify plant performance, and control OECD soil (OECD 1984) to verify worm performance. All treatments were replicated five times and followed a randomized block design. Plant and worm studies each included 70 test units (1 reference × 2 species × 2 clay content × 5 replicates) + (3 TNT treatments × 2 species × 5 replicates) + (2 TNT/clay treatments × 2 species × 5 replicates). Plant and worm studies each included 10 control units (1 control × 2 species × 5 replicates).

Table 2
Characteristics of the Soil Mixtures Prior to Incubation. Mean Values and Standard Deviations (N = 5 for Explosives, N = 3 for Other Characteristics)

Characteristic	Soil Mixtures						
	Reference[1] 0	Reference[1] 0 + clay[2]	Low-TNT[3] 5	Low-TNT[3] 5 + clay	Medium-TNT[3] 10	Medium-TNT[3] 10 + clay	High-TNT[3] 18
Explosives (mg kg^{-1} DW)							
TNT	<1.684	<1.684	5.40±2.76	4.33±3.36	10.30±33.40	8.90±4.16	18.02±8.06
2ADNT	<3.043	<3.043	0.95±0.93	0.96±0.16	2.70±1.86	1.12±1.05	3.69±1.43
4ADNT	<1.225	<1.225	<1.225	<1.225	0.26±0.59	<1.225	0.37±0.34
TNB	<1.000	<1.000	<1.000	0.67±0.77	6.86±9.99	0.75±1.02	2.04±1.08
RDX	<3.122	<3.122	8.57±0.73	11.45±3.04	59.05±11.21	59.34±12.31	153.86±4.22
HMX	<1.913	<1.913	1.54±0.12	1.72±0.40	6.51±0.13	7.48±1.45	17.15±0.56
Nutrients (mg kg^{-1} DW)							
Nitrate-N	117.93±15.71	94.91±1.66	86.30±0.75	88.00±11.45	68.86±11.30	74.21±0.76	79.35±1.60
Infinite-sink P	0.98±0.24	1.89±0.31	1.35±0.40	1.80±0.14	1.46±0.40	4.67±1.45	7.63±2.08
Other							
pH_{water}	6.51±0.04	6.53±0.02	6.61±0.01	6.56±0.02	6.57±0.01	6.55±0.02	6.15±0.13
OM (% DW)	5.22±0.06	5.25±1.12	5.01±0.22	4.63±0.64	4.51±0.25	3.78±0.08	3.76±0.11
DW (% FW)	90.55±0.15	90.48±0.14	91.95±0.07	90.46±0.48	91.59±0.56	89.82±0.37	89.91±0.65
BD (g DW mL^{-1})	2.17±0.22	2.47±0.12	2.40±0.24	1.92±0.16	1.99±0.14	2.10±0.27	1.96±0.04

Note: Abbreviations: BD, bulk density; FW, fresh weight; OM, Organic matter.
[1] Clean NOP soil.
[2] Clean clay, used in amendments of 30% w/w.
[3] Target TNT concentrations, in mg kg-1 DW.

The following responses were measured:

- For plants:
  - Toxicity, as measured by the plant biomass formed in 55 days in g DW m^{-2}.
  - Accumulation, as measured by the plant tissue-explosives concentrations accumulated in 55 days in mg kg^{-1} DW.
- For worms:
  - Toxicity, as measured in *E. fetida* by the biomass of 10 adult worms in g DW $unit^{-1}$ and the number per unit after 28 days of incubation.
  - Toxicity, as measured in *E. crypticus* as number per unit after 28 days of incubation and as the number of juveniles after 42 days of incubation.
  - Accumulation, as measured by the worm tissue-explosives concentrations accumulated in 28 days in mg kg^{-1} DW.

Soils

Three soils were mixed for the tests. The TNT-contaminated and reference soils both originated from the Nebraska Ordnance Plant (NOP), and a clay soil was from Vicksburg, MS. The NOP is located in Saunders County, Nebraska, near the town of Mead in an area referred to as Todd Valley. The soils in this area are of the Sharpsburg–Fillmore association, comprised of mostly Sharpsburg silty loam on well-drained locations. These soils were collected in 1996 from the NOP and stored in 55-gallon drums at room temperature until use. The TNT-contaminated soil was excavated from an area near former ordnance loading sites (Load Line 2A) and contained 171 mg TNT kg^{-1} DW, and 1513 mg RDX kg^{-1} DW, 150 mg HMX kg^{-1} DW, and 23 mg TNB kg^{-1} DW. Part of this soil was used in tests evaluating biomass production and explosives residues in agricultural crops (Price et al. 2002). The reference soil was excavated from a wooded area close to Load Line 2A and contained no detectable explosives levels. The uncontaminated clay soil was high in clay and organic matter. The Sharkey clay soil was also collected in 1996 and stored in a 55-gallon drum at room temperature until use (Table 1).

The three soils were spread and dried in the greenhouse to reach a moisture content of 5 to 10 percent. The explosives-contaminated soil was crushed and thoroughly mixed. During the latter process, about 90 percent of the TNT was degraded, as evaluated by determining the TNT concentration before and after drying/crushing. The reference and clay soils were ground to pass a 2-mm sieve. The properties of the three soils are presented in Table 1. The soil dilution series was constructed by mixing different amounts of explosives-contaminated soil with reference soil (Table 1, Table 2). Clay contents were increased by adding 30 percent (on a weight basis) of the clay-source soil in cases where an enhanced adsorptive capacity of the soil mixtures was desired. A clay-amended soil TNT concentration of 18 mg kg^{-1} DW was not included in the tests, because insufficient TNT-contaminated soil was available.

Plant Tests

For each *L. perenne* unit, 0.230 g of seeds (200) were weighed and placed on top of 1 L [1580 g fresh weight (FW)] of the appropriate soil mixture contained in 2-L plastic pots. For each *M. sativa* unit, 0. 201 g of seeds were planted. Plants were cultivated in a greenhouse at the Waterways Experiment Station, Vicksburg, MS. The pots were watered daily with reverse osmosis (RO) water to maintain the soil at a moisture level of 36 percent (field capacity was 38 percent). A moisture level at field capacity allows maximum mobility of contaminants in soil solution. Plants were amended with slow-release Osmocote fertilizer 10 days after onset of the experiment to attain target levels of 352 kg N ha^{-1}, 59.2 kg P ha^{-1}, and 331.9 kg K ha^{-1}, commonly used for pastures (Best and Jacobs 2001). They were harvested after 55 days of cultivation. Seeds germinated synchronously, as was verified before the onset of the tests.

Animal Tests

For *E. fetida*, 10 worms were placed on top of 100 g FW of the appropriate soil mixture contained in a 250-mL glass Mason jar. These animals were cultivated under continuous fluorescent illumination at 20 °C and were harvested after 28 days (ASTM 1998). For *E. crypticus*, 10 worms were placed on top of 2 g FW of the appropriate soil mixture contained in a 5-mL petri dish. These animals were cultivated in darkness at 16 °C. Adults were counted and removed after 28 days, and juveniles were estimated after 42 days. Estimates were done by counting four representative subsamples per unit. All units were moistened regularly with RO water.

Plant and Worm Harvesting and Sample Preparations

Fifty-five days after seeding, *L. perenne* and *M. sativa* were harvested in preparation for tissue analysis for explosives. Above- and below-ground plant portions were separated using stainless steel scissors. The plant tissues were washed in RO water to remove dust and soil particles, blotted as dry as possible, and weighed.

Twenty-eight days after inoculation, *E. fetida* and *E. crypticus* specimens were removed from their cultivation units. *E. fetida* worms were rinsed in RO water, weighed, placed on wet laboratory towels for 24–36 hr to purge ingested soil particles, and reweighed. *E. crypticus* worms were counted.

After collecting, washing, blotting, weighing, purging, and re-weighing were completed, plant and worm tissues were placed in plastic Ziploc bags and frozen at –80 °C. Subsamples were used to determine dry weight. Dry weight (plants and worms) was determined by drying the fresh material in a forced-air oven to constant weight (105 °C). To determine explosives in plant and worm tissues and in soil, modifications to method 8330 for soils (USEPA 1992) were used, as described below.

Extractions and Explosives Analyses

Plant extracts were prepared from freshly ground materials. Worm extracts were prepared from freeze-dried materials, because extracts from fresh materials with and without heating to remove water before extraction with acetonitrile yielded far lower recoveries of spiked TNT, 2ADNT, and 4ADNT than extracts from freeze-dried materials. Soil extracts were prepared from fresh material. Only three of the five replicate samples of each treatment were extracted. This was done because variations in biomass were expected to be larger than those in explosives concentrations; this also limited analytical costs.

Plants were clipped into small pieces and mixed. Subsamples for extraction were homogenized by grinding them in liquid nitrogen. Two-gram FW portions were spiked with 4-nitrotoluene (4NT) as an internal standard for recovery (50 µL of a 1-mg mL^{-1} solution), heated at 100 °C to remove water, and extracted in 5-mL acetonitrile by an 18-h sonication in a water-cooled bath at 15 °C. The extracts were freed from particles by centrifugation for 10 min at 2,000 g. HPLC analysis of the extracts was performed after cutting the supernatants 1:1 with Millipore-filtered RO water, recentrifugation, and clean-up over a 0.5-g Florisil column.

The worm extraction procedure included freeze-drying of aliquots equivalent to 0.7 g FW in bead-beater vials, amendment with 0.8 mL of acetonitrile, pulverizing by two successive cycles of 1-min bead-beating at room temperature (22–24 °C), and sonication for 1 h at 15 °C. These extracts were freed from particles by centrifugation for 10 min at 2,000 g. HPLC analysis of the extracts was performed after cutting the supernatants 1:1 with Millipore-filtered RO water, recentrifugation, and clean-up over a 0.45-µm polytetrafluoroethylene (PTFE) disk.

Just before incubation, each soil mixture was analyzed for explosives, and other chemical and physical characteristics, in triplicate. At the end of the incubation, one replicate of each ryegrass soil unit was screened for TNT residues. For the determination of explosives, 2 g FW was extracted in 10 mL of acetonitrile by 18-h sonication at 15 °C, clean-up over a Florisil column, and 10× concentration.

The plant, worm, and soil extracts of the samples in which the highest explosives levels were expected were first screened for the presence of all explosives listed by USEPA Method 8330 (USEPA 1992). After the explosives in these extracts were identified, only the relevant explosives were determined in all other extracts. The latter compounds were usually TNT, 2ADNT, 4ADNT, RDX, and HMX. In the spiked samples 4NT was also determined, and in the source soil samples TNB was determined.

Detection limits and recoveries for several target compounds spiked on plants, worms, and soil directly before extraction varied with compound. Method detection level (MDL) in mg kg^{-1} DW and recoveries were:

- **In freshly ground plant tissues:**
  MDL: TNT 0.081, 2ADNT 0.103, 4ADNT 0.161, 4NT 0.314, RDX 0.142, HMX 0.110 mg kg^{-1} DW
  Recovery: TNT 46.4, 2ADNT45.3, 4ADNT 41.9, 4NT 25.9, RDX 87.2, HMX 85.0 percent
- **In freeze-dried worm tissues:**
  MDL: TNT 1.174, 2ADNT 1.750, 4ADNT 1.773, RDX 2.176, HMX 1.645 mg kg^{-1} DW.
  Recovery: TNT 80.1, 2ADNT 88.2, 4ADNT 59.9, 4NT 25.9, RDX 83.3, HMX 60.1 percent
- **In freshly ground soil:**
  MDL: TNT 1.684, 2ADNT 3.043, 4ADNT 1.225, RDX 3.122, HMX

1.913 mg kg^{-1} DW
Recovery: TNT 10.4, 2ADNT 15.1, 4ADNT 4.8, RDX 99.5, HMX 12.3 percent.

Typical HPLC chromatograms of extracts of *L. perenne* and *E. fetida* exposed to TNT-contaminated soil are presented in Figure 1.

Other Soil Analyses

Moisture content was determined by drying at 105°C in a forced-air oven until constant weight. Concentrations of organic matter were determined by loss on ignition at 550 °C, and bulk density was determined volumetrically (Allen et al. 1974). pH_{KCl} was measured with a pH meter (Beckman Model PHI40, Fullerton, CA) in a 1-M KCl solution in a fresh-soil-to-liquid ratio of 1:2.5 (w/v). pH_{KCl} was converted to pH_{water} using a regression equation of $pH_{water} = 0.677 \times pH_{KCl} + 2.35$ (Best and Jacobs 2001).

Nitrate-N was determined after conversion to ammonia, using a Hach spectrophotometer DR/2000 (Hach 1992). Ammonia-N was determined spectrophotometrically (HACH DR 4000 U spectrophotometer, Hach Company, Loveland, CO) according to EPA method 350.2 M (USEPA 1983). Plant-available P was determined as infinite-sink phosphorus (P_i) concentration according to Van der Zee et al. (1987). The P_i determination measures both P fractions that are relatively rapidly adsorbed to Fe and Al and P fractions that are relatively slowly precipitating. Total-K was determined by extraction in a hydrochloric acid/oxalic acid mixture and measurement of the cesium chloride complex spectrophotometrically according to Houba et al. (1995).

Statistics

Statistical analyses were conducted with the software STATGRAPHICS Plus for Windows 3 (Manugistics, Rockville, MD; 1997).

Linear correlation between the initial explosives concentrations in the soil mixtures was tested using the Pearson product moment correlation procedure.

Normal distribution of the data was tested with the Shapiro-Wilk's test. The data were ln-transformed because their distributions significantly deviated from a normal distribution.

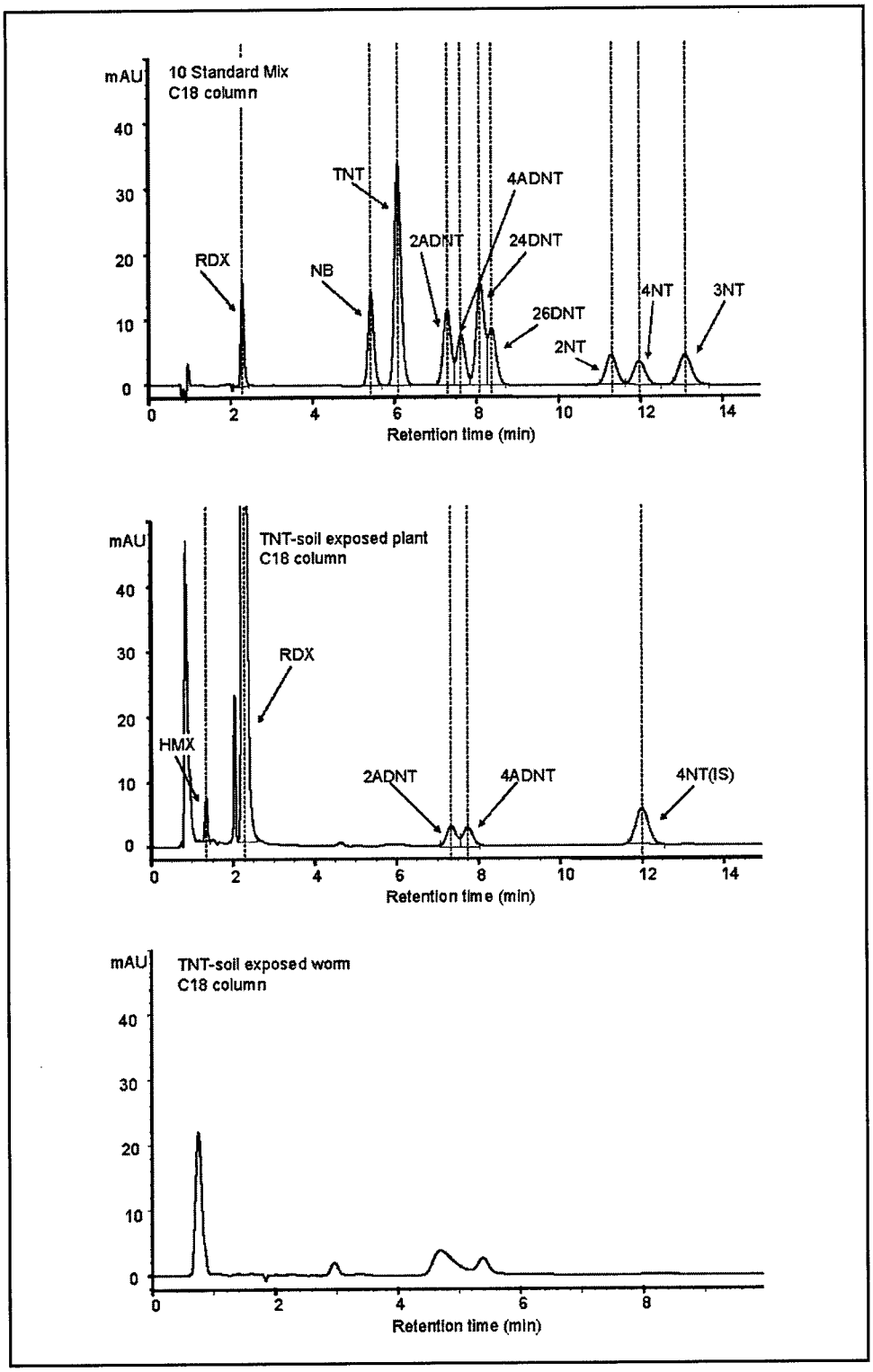

Figure 1. Typical HPLC chromatograms of extracts of *L. perenne* and *E. fetida*, exposed to TNT-contaminated, aged soil. A chromatogram of a 10-standard mixture is presented for reference. IS, internal standard

Analysis of variance (ANOVA) of the ln-transformed data was conducted with the explosives concentration of the soil mixture as the main factor and clay amendment as covariate. This analysis was expanded with a multiple range test using the Fisher's least significant difference procedure. The p-value in the ANOVA is a measure of the significance of the analysis; it was set at a 95-percent confidence level (p value of ≤ 0.05). In this analysis the sum of plant tissue 2ADNT and 4ADNT concentrations was included as TNT-derived metabolites (recalculated on a molar basis).

Linear regression analyses were conducted of the ln-transformed data, using the least squares method. For this analysis, zero values for soil explosives levels were replaced by half of the detection levels. The p-value in the regression model was set at a 95 percent confidence level (p value of ≤ 0.05) unless stated otherwise. The R^2-value of the regression model indicates the proportion of the variance explained by the model. Also, in this analysis, the sum of plant tissue 2ADNT and 4ADNT concentrations was included as TNT-derived metabolites (recalculated on a molar basis).

3 Results

Soil Mixtures

The explosives concentrations in the seven soil mixtures ranged from non-detectable to 18 mg TNT kg^{-1} DW, 154 mg RDX kg^{-1} DW, and 17 mg HMX kg^{-1} DW at the beginning of the incubations (Table 2). The TNT, RDX, and HMX concentrations were significantly correlated, with correlation coefficients between TNT, RDX, and HMX of ≥97 percent. After the 55-day incubation with plants, the TNT levels were below the detection level of 1.684 mg kg^{-1} (non-extractable).

Toxicity and Bioaccumulation in Plants

Short-term exposure tests

Acute toxicity was not observed after 10 days exposure, and seeds had germinated in all plant units. Germination in *L. perenne* seeds was inhibited at TNT concentrations ≥100 mg kg^{-1}. Germination in seeds on soil spiked at 100 mg TNT kg^{-1} DW was similar to that on aged soil containing 171 mg TNT kg^{-1} DW, i.e. 78 and 76 percent, respectively. Germination in *M. sativa* seeds fluctuated between 83 and 90 percent (Table 3). The effect of TNT exposure on germination was not significant, as demonstrated by ANOVA of the ln-transformed data (for *L. perenne* a p-value of 0.398 was found, and for *M. sativa* a p-value of 0.329).

Long-term exposure tests

After 55 days of exposure, *L. perenne* biomass was significantly lower on the TNT-contaminated soil mixtures than on the reference soil, but plants survived on all soil mixtures (Table 4).

Table 3
Seed Responses to 10 Days of Exposure to TNT-Spiked and TNT-Contaminated, Aged Soil. Germination of the Seeds on Control Soils Were: *L. perenne* on Non-Spiked Control-OECD Soil 86.4±10.8, and on Control Potting Soil 72.8±5.2 Percent; *M. sativa* on Non-Spiked Control-OECD Soil 83.2±5.2, and on Control Potting Soil 84.8±5.9 Percent. ANOVA Results[1] Are Listed

Soil	TNT concentration (mg kg^{-1} DW)	Germination (%)	
		L. perenne	*M. sativa*
OECD, spiked	0	80.0±16.2a	89.6±3.6a
OECD, spiked	10	88.0±5.7a	84.0±6.9a
OECD, spiked	50	84.0±10.2a	83.2±7.2a
OECD, spiked	100	77.6±9.2a	89.6±6.1a
NOP, aged	171	76.0±11.7a	90.4±8.3a
ANOVA[1]			
P-value		0.398	0.329
MS		0.020	0.007
F-ratio		1.05	1.20

[1] ANOVA results of ln-transformed data, using target TNT concentration as factor. Ln-transforms of the values that are followed by the same letter are not significantly different according to Fisher's least significant difference procedure.

Shoot and root biomass decreased with soil TNT concentration. This effect was significant at the 95 percent confidence level for shoots but at a lower (90 percent) confidence level for roots (Table 4). Generally less plant biomass was produced on the clay-amended soil mixtures than on the nonamended soils, but the effect of clay amendment was not statistically significant (Table 4). Plant biomass produced on reference soil was similar to that on the control soil, confirming that the test soil without contamination supported adequate plant growth (Table 4).

Shoot and root biomass were related to soil TNT concentration using linear regression of the ln-transformed values. Regression equations relating biomass to soil TNT concentration with clay amendment as the cofactor usually explained more of the variability in the data set (i.e., they yielded higher r^2) than regression equations relating biomass to soil TNT concentration alone (Table 5; Figure 2). Two linear regression equations describing the relationships between ln shoot biomass and soil TNT concentration were found. The first equation takes clay content into account, i.e., $LnY = 3.411 - 0.094X - 0.013CLAY$; $p<0.001$, $r^2 = 49$ percent. In this equation Y = shoot biomass, X = soil TNT concentration, and CLAY = 30 percent soil. The second equation does not take clay content into account, i.e. $LnY = 3.175 - 0.085X$; $p<0.001$, $r^2 = 43$ percent. Using the regression equation that explained the highest portion of the variability in the data set, i.e., $Ln\ Y = 3.411 - 0.094X - 0.013\ CLAY$, a 55-d EC50 of 7.3 mg TNT kg^{-1} and a 55-d EC20 of 2.4 mg TNT kg^{-1} soil DW were found for *L. perenne*. The linear regression equations describing the relationships between root biomass and soil TNT concentration had p-values that were significant

Table 4
Biomass and Tissue Explosives Concentration of Shoots and Roots of *L. perenne* in Response to 55 Days of Exposure to TNT-Contaminated Soil. Mean Values and Standard Deviations Are Shown (Biomass N = 5; Explosives Concentrations N = 3). Shoot and Root Weights of the Control Plants Were: 43.92±5.87 g DW m^{-2} and 19.60±2.26 g DW m^{-2}, Respectively. ANOVA Results[1] Are Listed.

Soil Mixture	Biomass (g DW m^{-2})	Explosives Concentration (mg kg^{-1} DW)			
		2ADNT	4ADNT	RDX	HMX
Shoots					
Reference	41.84±12.38a	<0.103	<0.161 a	<0.142 a	< 0.110 a
Reference, clay	27.55±13.38a	<0.103	<0.161 a	<0.142 a	< 0.110 a
5-mg kg^{-1} TNT	17.70±12.46b	<0.103	<0.161 a	1128.7±57.5 b	32.0±4.4 b
5-mg kg^{-1} TNT, clay	12.75±9.89 b	<0.103	<0.161 a	1037.3±366.0 b	27.5±3.5 b
10-mg kg^{-1} TNT	8.48±2.08 bc	7.2±4.2	7.5±5.5 b	5847.0±3137.9 c	119.3±83.1c
10 mg kg^{-1} TNT, clay	9.64±3.94 b	9.7±1.1	9.8±3.9 b	4587.6±556.9 c	84.0±10.1c
18-mg kg^{-1} TNT	7.48±2.00 c	18.8±16.2	19.3±18.8b	2947.5±2881.5 b	62.3±72.7bc
ANOVA[1]					
P-value	<0.001		<0.001	<0.001	<0.001
MS	3.90		69.95	149.49	58.10
F-ratio	12.93		254.67	488.93	108.08
Roots					
Reference	8.25±2.01 a	<0.103	<0.161 a	<0.142 a	< 0.110 a
Reference, clay	6.59±3.34 a	<0.103	<0.161 a	<0.142 a	< 0.110 a
5-mg kg^{-1} TNT	2.73±1.39 b	23.7±26.4	33.5±38.8a	466.5±208.6 b	21.7±11.2 b
5-mg kg^{-1} TNT, clay	2.84±3.36 b	2.9±5.0	2.8±4.8 a	612.8+278.1 b	26.8+14.0 b
10-mg kg^{-1} TNT	2.27±0.78 b	21.7±9.0	20.8±8.7 a	2127.8±1547.3c	80.9±24.2 b
10 mg kg^{-1} TNT, clay	2.32±1.06 b	17.1±15.6	21.7+19.9a	1740.6+1142.7c	41.5±36.8 b
18-mg kg^{-1} TNT	2.83±3.11 b	44.9±32.4	61.0±47.2a	2227.2±1800.4bc	76.9±66.7 b
ANOVA[1]					
P-value	0.005		0.037	<0.001	<0.001
MS	7.03		37.72	124.37	42.45
F-ratio	5.28		5.61	172.65	9.34

[1] ANOVA results of ln-transformed data, using target explosives concentration as factor and clay amendment as covariate. Ln-transforms of the values that are followed by the same letter are not significantly different according to Fisher's least significant difference procedure. In this analysis, the sum of plant tissue 2ADNT and 4ADNT concentrations was included as TNT-derived metabolites (recalculated on a molar basis).

at a lower confidence level of 90 percent, but explained only a low portion of the variability in the data set, i.e., up to 18 percent. For the latter reason, these equations were not considered accurate enough to predict root biomass from soil TNT concentration.

Table 5
Linear Regressions of ln-Transforms of *L. perenne* Response Values and Target Explosives Concentrations in the Soil Mixtures, ln Y = a + bX + cCLAY (Y = Plant Response; X = Target Explosives Concentration Soil Mixture; CLAY = Current Clay Amendment, i.e., 30 Percent, w/w). Values for Biomass Are in g DW m^{-2}; Values for Explosives and Metabolite Concentrations (the Latter for TNT Only) in Plant Tissues Are in mg kg^{-1}DW. Biomass and Tissue TNT-Metabolites Were Regressed on Soil TNT Concentration, Tissue RDX and HMX Concentrations on Soil RDX and HMX Concentration, Respectively

Response	Statistic Parameter			Statistic Fitted Model	
	Estimated Value	Standard Error	p-value	p-value	R^2 (percent)
Shoots					
Biomass					
a	3.411	0.195	<0.001	<0.001	48.6
b	–0.094	0.017	<0.001		
c	–0.013	0.007	0.065		
Tissue TNT-metabolites					
a	–4.445	1.238	0.004	<0.001	69.7
b	0.514	0.100	<0.001		
c	0.029	0.032	0.382		
Tissue RDX					
a	7.363	0.498	<0.001	0.697	5.8
b	0.004	0.005	0.406		
c	0.005	0.018	0.788		
Tissue HMX					
a	0.222	1.045	0.834	0.037	30.6
b	0.304	0.109	0.012		
c	0.020	0.041	0.629		
Roots					
Biomass					
a	1.699	0.244	<0.001	0.050	18.1
b	–0.055	0.024	0.029		
c	–0.013	0.009	0.147		
Tissue TNT-metabolites					
a	2.796	1.664	0.118	0.041	41.2
b	0.111	0.134	0.425		
c	–0.100	0.043	0.040		
Tissue RDX				0.503	10.8
a	6.335	0.551	<0.001		
b	0.007	0.006	0.253		
c	0.007	0.020	0.744		
Tissue HMX					
a	0.432	1.221	0.727	0.233	14.9
b	0.209	0.127	0.118		
c	–0.007	0.048	0.887		

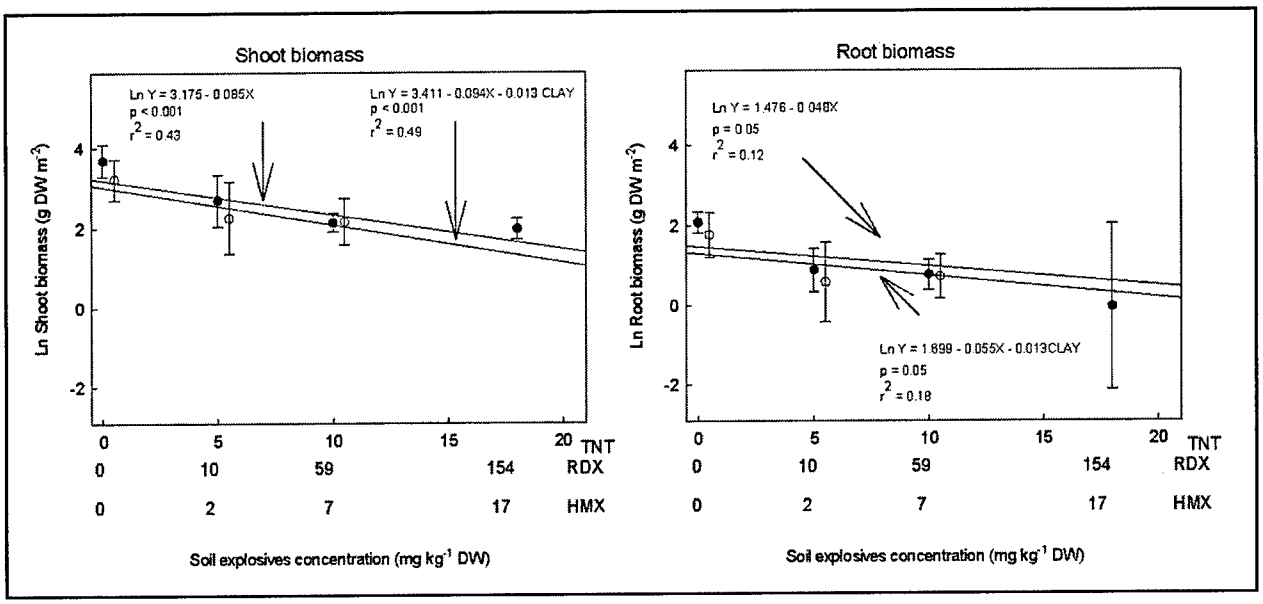

Figure 2. Linear regressions of ln-transforms of *L. perenne* biomass values and target explosives concentrations in the soil mixtures. Y, plant response; X, target explosives concentration soil mixture; CLAY, clay amendment of 30 percent w/w

TNT was not recovered from the plant material (Table 4). However, low concentrations of the TNT metabolites 2ADNT and 4ADNT up to 19 mg kg^{-1} DW were found in the shoots of plants exposed to the 10-mg-TNT and 18-mg-TNT soil mixtures. Concentrations of 2ADNT and 4ADNT in roots usually exceeded those in shoots (up to 61 mg kg^{-1} DW) and were detectable in all plants exposed to TNT-contaminated soils. Of the other explosives and metabolites initially present in the soil mixtures, both RDX and HMX were recovered from the plant material, but TNB was not. Both RDX and HMX accumulated in the plants to an extent that greatly exceeded the concentrations in the soil mixtures: RDX mainly in shoots and HMX in shoots and roots (Table 4).

The TNT metabolite concentrations in the shoots increased significantly with the initial TNT concentration in the soil mixtures (Table 5, Figure 3). Two linear regression equations describing the relationships between TNT metabolites in shoots and soil TNT concentration were found. The first equation takes clay content into account, i.e., $LnY = -4.445 + 0.514X + 0.029CLAY$; $p<0.001$, $r^2 = 70$ percent. In this equation Y = TNT metabolites concentration in shoots, X = soil TNT concentration, and CLAY = 30 percent soil. The second equation does not take clay content into account, i.e. $LnY = -3.780 + 0.482X$; $p<0.001$, $r^2 = 67$ percent. These equations can be used to predict the TNT metabolite concentrations in shoots from the TNT concentration in the soil to which the plants are exposed. Using the regression equation that explained the highest portion of the variability in the data set, i.e., $Ln\ Y = -4.445 + 0.514X + 0.029$ CLAY, an accumulation of 122 mg kg^{-1} TNT metabolites would be expected in *L. perenne* shoots exposed for up to 55 days to 18 mg TNT kg^{-1} soil DW.

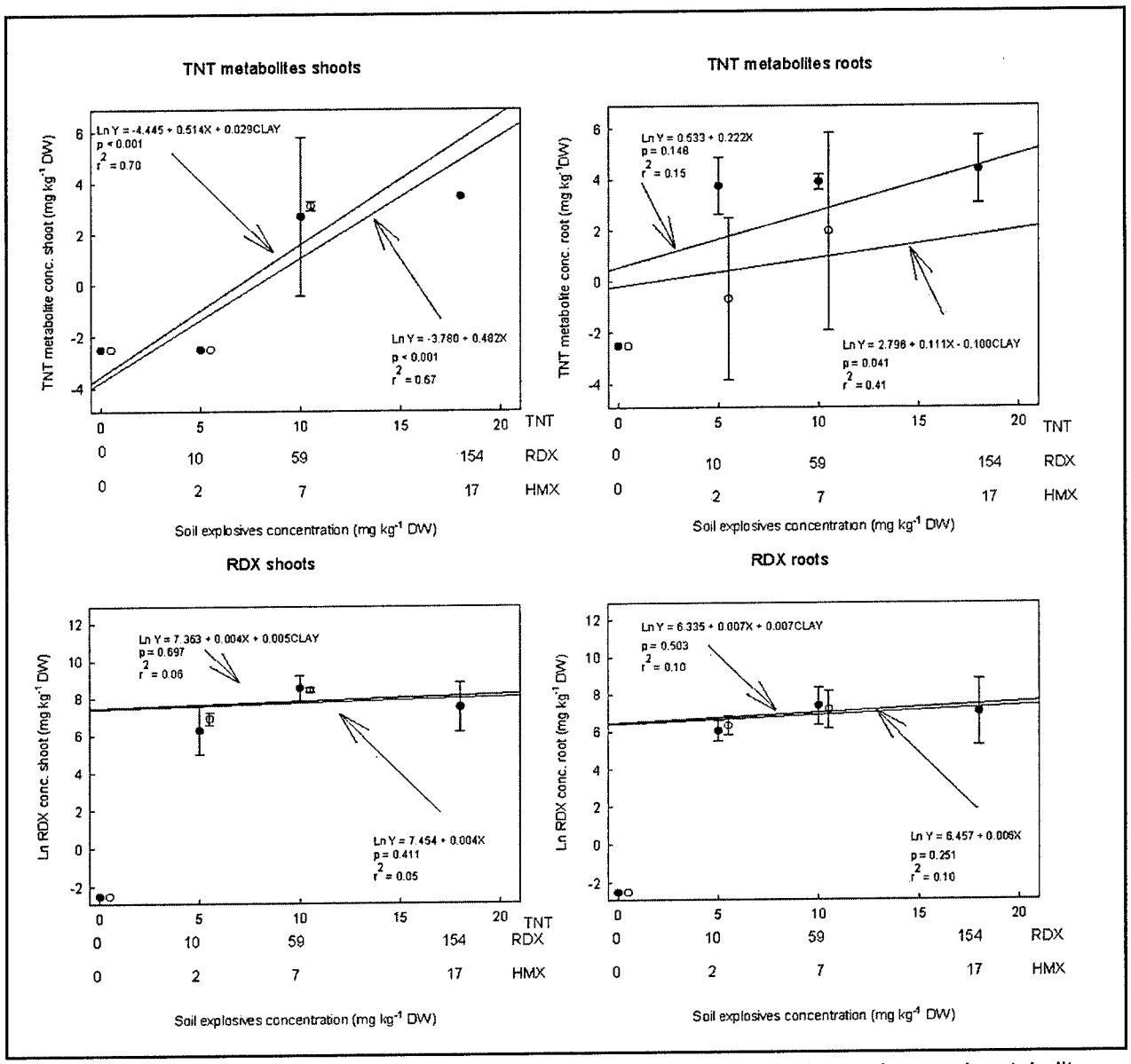

Figure 3. Linear regressions of ln-transforms of *L. perenne* tissue explosives values and metabolite concentrations, and target explosives concentrations in the soil mixtures. Y, plant response; X, target explosives concentration soil mixture; CLAY, clay amendment of 30 percent w/w (Continued)

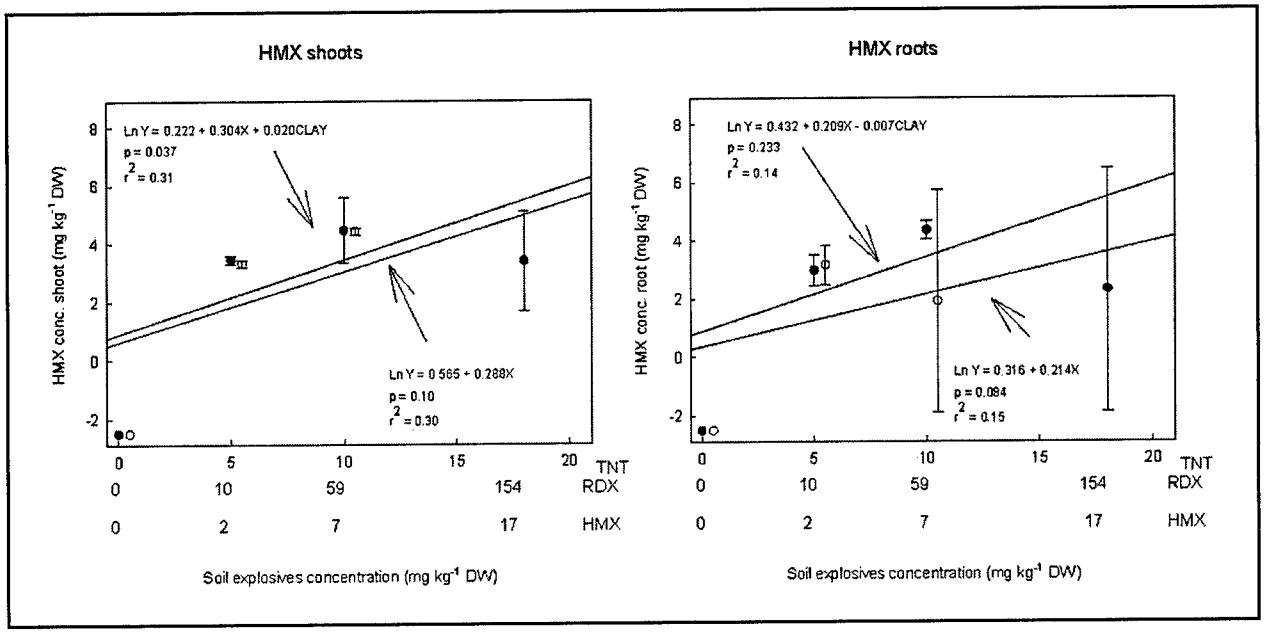

Figure 3. (Concluded)

The HMX concentrations in the shoots also increased significantly with initial HMX concentrations in the soil, but in this case only a small part of the data set was explained by the regression equation, i.e. 30–31 percent (Table 5, Figure 3). No significant linear regression equations between TNT metabolite concentrations in roots and initial soil TNT concentration, RDX concentrations in plant parts and those in soil, and HMX concentration in roots and those in soil were found (Table 5, Figure 3).

Because only the effects of the soil-TNT concentrations on *L. perenne* shoot biomass and TNT-metabolite concentrations were significant, the toxicity of the soil was attributed mainly to the contamination by TNT. However, the other explosives in the soil may have contributed also.

After 55 days of exposure, all *M. sativa* plants exposed to aged soil-TNT concentrations ≥5.4 mg kg^{-1} DW had died. However, substantial biomass was produced on the reference soil either amended or not amended with clay (Table 6). This observation leads us to a conservative estimate of ≤2.7 mg TNT kg^{-1} soil as EC50 for this species. This plant material was not further analyzed.

Table 6
Biomass of *M. sativa* Plants in Response to 55 Days of Exposure to TNT-Contaminated Soil. Mean Values and Standard Deviations (Biomass N = 5). Weight of *M. sativa* Control Plants Was 9.42 ± 2.28 g DW m^{-2}

Soil Mixture	Biomass (g DW m^{-2})
Plants	
Reference	6.06±1.51
Reference, clay	6.37±1.77
5-mg kg^{-1} TNT	2.14±1.43
5-mg kg^{-1} TNT, clay	1.83±1.86
10-mg kg^{-1} TNT	0
10 mg kg^{-1} TNT, clay	0
18-mg kg^{-1} TNT	0

Toxicity and Bioaccumulation in Worms

Short-term exposure tests

Acute toxicity was observed in both worm species after 14 days of exposure. TNT concentrations of ≥100 mg kg^{-1} were lethal for *E. fetida*, while at a concentration of ≥10 mg TNT kg^{-1}, 50 percent of the *E. crypticus* individuals died. Because both species survived in uncontaminated control soils, spiked or not spiked with the same solvent as used to spike the TNT-contaminated soils, worm death was attributed to the TNT exposure (Table 7). The methanol used for spiking decreased worm survival by >30 percent. The effect of TNT exposure on the number of surviving worms was not significant, as found by ANOVA of the ln-transformed data (for *E. fetida* a p-value of 0.580 was found, and for *E. crypticus* the p-value was 0.202).

Long-term exposure tests

After 28 days of exposure, mortality was observed again in both worm species, but at lower TNT-levels than in the range-finding experiment. TNT concentrations of ≥18 mg kg^{-1} were lethal for *E. fetida*, and of ≥4.3 mg kg^{-1} were lethal for *E. crypticus*. Worm biomass and number of individuals of *E. fetida* decreased significantly with soil TNT concentration, and all worms had died in the soil containing 18 mg TNT kg^{-1} DW (Table 8). The effect of clay amendment was not significant. Worm biomass on the reference soil was similar to that on the control soil, confirming that the test soil supported adequate worm growth (Table 8).

Table 7
Worm Responses to 14 Days of Exposure to TNT-Spiked and TNT-Contaminated, Aged Soil. ANOVA Results[1] Are Listed. Survival on Control Soils Was: *E. fetida* on Non-Spiked Control-OECD Soil 100.0±0 Percent Unit^{-1}; *E. crypticus* on Non-Spiked Control-OECD Soil 58.0±20.5 Percent Unit^{-1}

Soil	TNT concentration (mg kg^{-1} DW)	Survival (%)	
		E. fetida	*E. crypticus*
OECD, spiked	0	62.0±11.0a	44.0±20.7a
OECD, spiked	10	76.0±23.0a	8.0±11.0a
OECD, spiked	50	58.0±37.0a	0±0a
OECD, spiked	100	0±0a	6.0±13.4a
NOP, aged	171	0±0a	0±0a
ANOVA[1]			
P-value		0.580	0.202
MS		0.053	0.594
F-ratio		0.57	1.88

[1] ANOVA results of ln-transformed data, using target TNT concentration as factor. Ln-transforms of the values that are followed by the same letter are not significantly different according to Fisher's least significant difference procedure.

Table 8
Biomass and Number of Individuals of *E. fetida* in response to 28 Days of Exposure to TNT-Contaminated Soil. Mean Values and Standard Deviations (N = 5). Weight of the Control Worms Was 0.791±0.075 g DW unit^{-1}. ANOVA Results[1] Are Listed. The Tissue Explosives Concentrations Were Below Detection

Soil Mixture	Worm Biomass (g DW unit^{-1})	Worm Number (N unit^{-1})
Reference	0.76 ± 0.05a	9.8 ± 0.4a
Reference, clay	0.78 ± 0.09a	9.4 ± 1.3a
5-mg kg^{-1} TNT	0.71 ± 0.09a	9.2 ± 1.3a
5-mg kg^{-1} TNT, clay	0.68 ± 0.11a	8.6 ± 0.9a
10-mg kg^{-1} TNT	0.38 ± 0.08b	5.8 ± 3.5b
10-mg kg^{-1} TNT, clay	0.68 ± 0.11b	7.4 ± 2.0b
18-mg kg^{-1} TNT	0c	0c
ANOVA[1]		
P-value	0.014	<0.001
MS	20.98	23.84
F-ratio	486.63	253.25

[1] ANOVA results of ln-transformed data, using explosives class as factor and clay amendment as covariate. Ln-transforms of the values that are followed by the same letter are not significantly different according to Fisher's least significant difference procedure.

Worm biomass and number decreased significantly with target soil-TNT concentration as found by linear regression of the ln-transformed values (R^2 values of 72 and 70 percent, respectively; Table 9).

Table 9
Linear Regressions of ln-Transforms of *E. fetida* Response Values and Target TNT Concentrations in the Soil Mixtures, ln Y = a + bX +cCLAY (Y = Worm Response, X = Initial TNT Concentration Soil Mixture; CLAY = Current Amendment, i.e. 30 Percent, w/w). Values for Biomass Are in g DW unit^{-1}; Values for Number Are in N unit^{-1}

Response	Statistic Parameter			Statistic Fitted Model	
	Estimated Value	Standard Error	p-value	p-value	R^2 (percent)
Worm biomass					
a	0.042	0.273	0.879	<0.001	71.8
b	–0.194	0.024	<0.001		
c	0.019	0.010	0.054		
Worm number					
a	2.652	0.297	<0.001	<0.001	70.2
b	–0.207	0.027	<0.001		
c	0.017	0.011	0.125		

Two linear regression equations describing the relationships between ln worm biomass and soil TNT concentration were found (Figure 4; Table 9). The first equation takes clay content into account, i.e., $LnY = 0.042 - 0.194X + 0.019CLAY$; $p<0.001$, $r^2 = 72$ percent. In this equation Y = worm biomass, X = soil TNT concentration, and CLAY = 30 percent soil. The second equation does not take clay content into account, i.e. $LnY = 0.382 - 0.207X$; $p<0.001$, $r^2 = 68$ percent. Using the regression equation that explained the highest portion of the variability in the data set, i.e., $LnY = 0.042 - 0.194X + 0.019CLAY$, a 28-d EC50 (or LC50) of 3.6 mg TNT kg^{-1} and a 28-d EC20 of 1.2 mg TNT kg^{-1} soil DW were found for *E. fetida*. Similarly, two equations were found to relate ln worm number to soil TNT concentration. The first equation was $LnY = 2.652 - 0.297X + 0.017CLAY$; $p<0.001$, $r^2 = 70$ percent. In this equation Y = worm number, X = soil TNT concentration, and CLAY = 30 percent soil. The second equation does not take clay content into account, i.e. $LnY = 2.945 - 0.219X$; $p<0.001$, $r^2 = 68$ percent. These equations yield values for EC50 and EC20 similar to those for worm biomass.

Because only the effect of the soil-TNT concentrations on *E. fetida* biomass was significant, the toxicity of the soil was attributed mainly to the contamination by TNT. However, the other explosives found in the soil may have contributed also.

None of the explosives and metabolites that had been identified in the soil mixtures prior to incubation were detected in *E. fetida*.

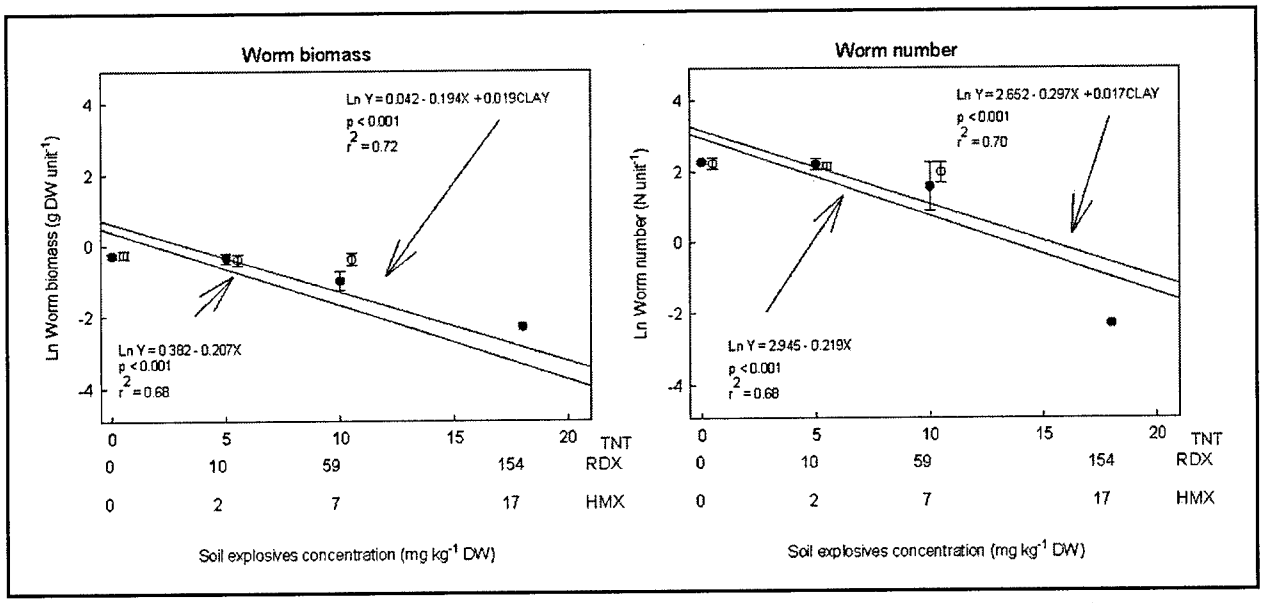

Figure 4. Linear regressions of ln-transforms of *E. fetida* biomass values, or number, and target explosives concentrations in the soil mixtures. Y, plant response; X, target explosives concentration soil mixture; CLAY, clay amendment of 30 percent w/w

After 28 days of exposure, *E. crypticus* survived only on the reference soils (data not shown). This observation leads us to a conservative estimate of ≤2.15 mg TNT kg^{-1} soil as EC50 for this species. This worm material was not further analyzed.

4 Discussion

Toxicity and Bioaccumulation in Plants and Worms

Our studies show that the potential toxicity of TNT from TNT-contaminated, aged soil to plants and worms depends on species and exposure duration, while in plants it is also influenced by life stage and bioavailability as modified by clay content.

Both plant species used, *L. perenne* and *M. sativa*, tolerated TNT concentrations up to 171 mg kg^{-1} DW during short-term exposure. During long-term exposure, both plant species were less tolerant of TNT, although *L. perenne* was more tolerant than *M. sativa*. A 55-d EC50 of 7.3 mg TNT kg^{-1} and a 55-d EC20 of 2.4 mg TNT kg^{-1} soil DW were derived for *L. perenne* by linear regression of the ln-transformed plant biomass values to soil-TNT concentration. A 55-d EC50 of <2.7 mg TNT kg^{-1} was estimated for *M. sativa* from the lethal soil-TNT concentration of 5.4 mg kg^{-1}. Among the worm species, *E. fetida* tolerated TNT concentrations up to 100 mg kg^{-1}, while 50 percent of the *E. crypticus* individuals died at a TNT concentration of 10 mg kg^{-1} and none survived exposure to higher concentrations during short-term exposure. During long-term exposure, both worm species were less tolerant of TNT, but *E. fetida* was more tolerant than *E. crypticus*. A 28-d EC50 of 3.6 mg TNT kg^{-1} and a 28-d EC20 of 1.2 mg TNT kg^{-1} soil DW were derived for *E. fetida* by linear regression the ln-transformed worm biomass values to soil-TNT concentration. The EC values for surviving number of individuals were similar. A 28-d-EC50 of ≤2.15 mg TNT kg^{-1} for *E. crypticus* was estimated from the lethal soil-TNT concentration of 4.3 mg kg^{-1}. Because biomass of *L. perenne* and of *E. fetida* decreased only significantly with the soil concentrations of TNT, but not with those of RDX or HMX, the soil toxicity was attributed mainly to contamination by TNT.

We observed that the duration of the exposure period of plants and worms to TNT- contaminated soil has profound effects on their responses, resulting in different estimates of harmful media-specific concentrations. Short-term (10- to 14-day) tests generated responses indicating a higher tolerance towards TNT than long-term (28- to 55-day) tests. Ten days of exposure of *L. perenne* and *M. sativa* seeds to TNT concentrations up to 171 mg kg^{-1} did not evoke an acute toxicity response, while a 55-day exposure of plants to TNT concentrations up to

18 mg kg^{-1} did. This difference may be explained by the following: (1) degradation of the spiked TNT during the period, allowing for evaporation of the solvent vehicle prior to incubation of the test organisms; (2) interference of TNT metabolites with plant metabolism in post-germination stages; and (3) insensitivity of seeds to TNT during early germination. Fourteen days of exposure of *E. fetida* and *E. crypticus* evoked acute toxicity responses in both organisms at higher TNT concentrations than 28 days of exposure. In this case the difference may also be attributed to degradation of the spiked TNT prior to the first contact between the test organism and the contaminant, as well as to interference of TNT metabolites with growth during longer incubation periods.

Amendment of the soil with clay affected plant growth in general, although not significantly, probably largely by changing nutrient and water availability, since plant biomass was generally lower on all clay-amended soil mixtures, including the reference soils.

Toxicity Screening Benchmarks

The LOECs based on the presently found EC20 values for plants and worms are considerably lower than the published screening benchmarks (Talmage et al. 1999). For plants the screening benchmark was 30 mg kg^{-1} for bush bean exposed to TNT-contaminated, aged soil, while the currently measured LOEC is 2.4 mg kg^{-1} for perennial ryegrass. The noted difference may be explained by differences in species-specific tolerance towards TNT (and possibly other explosives usually co-occurring with TNT in explosives-contaminated, aged soils) and exposure duration. For soil invertebrates the screening benchmarks ranged from 140 to 200 mg kg^{-1}, measured for earthworms, nematodes, and arthropods exposed to TNT-spiked soil, while the currently measured LOEC is 1.2 mg kg^{-1} for earthworms. The latter difference may be explained by the spiked soils and short exposure periods employed in the tests on which the published screening benchmark is based. This explanation is supported by the far-lower LOECs of 7 and 19 mg kg^{-1} found for earthworms exposed to TNT-contaminated soil aged for 14 days (Simini et al. 1995).

Bioconcentration and Biotransfer of Explosives

To predict the effects of contaminants on populations and in food chains, evaluating the biotransfer of the contaminant in the ecological groups of which the food chain is composed is important as a basis for potential trophic transfer.

Empirical data on the biotransfer of explosives in lower trophic levels such as plants and worms are extremely scarce, and in the absence of these data, ecological risk approaches are often based on linear regression equations derived from residues of organics other than explosives in vegetation and beef (Travis and Arms 1988). The latter approach assumes that the potential of a chemical to accumulate in an organism, i.e. the **bioconcentration factor (BCF)**, is defined as a chemical's concentration in an organism or tissue divided by its concentration

in food. **BCFs** in the current study were on average 1.9 (range 1.5–2.1) for TNT metabolites, 85 (range 19–131) for RDX, and 14 (range 4–21) for HMX in plant shoots. BCFs in worms could not be calculated. However, the **biotransfer factor (BTF)** is more useful in risk assessment, since chemical exposure to the organisms may occur through both food and water pathways. To relate tissue concentrations in the above-ground biomass of vegetation to soil concentrations of a contaminant, the following equation is used (Travis and Arms 1988): $\log B_v = 1.588 - 0.578 \log K_{OW}$, in which B_v is the biotransfer factor in herbaceous plant shoots and K_{OW} is the octanol–water partitioning coefficient of that particular contaminant. Similarly, to relate tissue concentrations in beef to soil concentrations of a contaminant, the following equation is used (Travis and Arms 1988): $\log B_b = -7.6 + \log K_{OW}$, in which B_b is the biotransfer factor in beef of that particular contaminant. Using these BTF equations, the expected explosives concentrations in plant shoots and worms exposed to the aged test soil of the current study containing 18 mg kg^{-1} TNT and accompanying explosives (153.8 mg RDX and 17.1 mg HMX kg^{-1}; Table 2 present study) were calculated. Expected concentrations in plant shoots were 19.1–82.9 mg TNT kg^{-1}, 1876.4 mg RDX kg^{-1}, and 470.3–607.1 mg HMX kg^{-1} plant DW. Expected concentrations in worms were in the 10^{-5} to 10^{-3} mg kg^{-1} DW range for TNT, RDX, and HMX. The explosives concentrations recovered from the plant shoots in the current study were in the calculated ranges for TNT metabolites and for RDX, but they were lower than calculated for HMX. The nondetectable explosives residue levels in the worms of the current study were in agreement with the calculated range, which is lower than the lowest explosives (metabolite) detection level we attained (>1.1 mg kg^{-1}).

A different approach to predicting bioaccumulation of hydrophobic organic contaminants (HOCs) from substrates in biota has been suggested and applied to contaminated sediments by MacFarland (1995). According to this approach, the theoretical bioaccumulation potential (TBP) is expressed as a probable organism tissue concentration of a chemical of interest, and it depends on a partition coefficient, termed BSAF. A BSAF is the ratio of lipid-normalized concentration in an organism to organic carbon-normalized concentration of the chemical in the substrate to which the organism is exposed:

$$TBP = BSAF(C_S/f_{OC})f_L$$

where

BSAF = biota-substrate accumulation factor

C_S = HOC concentration in whole substrate

f_{OC} = decimal fraction of organic carbon (OC) in substrate

f_L = decimal fraction of lipid in targeted organism.

The BSAFs for the explosives metabolites recovered in the *L. perenne* shoots of the current study have been calculated using a lipid concentration of 6.75 percent DW measured earlier (Best et al. 2004) and assuming an OC content of 5.8 percent organic matter (Jackson 1964). Thus, the following BSAFs were calculated in shoots of plants exposed to the soil mixture containing 18 mg TNT kg^{-1} DW: 0.078 for TNT-derived metabolites, 0.618 for RDX, and 0.118

for HMX. The BSAF for TNT itself is 0, because TNT was not recovered in the plants. BSAFs of explosives in worms could not be calculated since explosives residues were below detection. The currently found BSAF for TNT metabolites in *L. perenne* is lower than the BSAF of 0.1031, the latter being the average BSAF calculated for naphthalene in macrofauna by MacFarland. Naphthalene was selected for the comparison, since it has a log K_{OW} of 3.36, being closest to the log K_{OW} of TNT (1.6–2.7, Talmage et al. 1999) of HOCs for which published BSAF values exist. The currently found BSAF values for RDX and HMX are far higher than that for naphthalene. The BSAF values reported in this paper can be used for predicting the residues of explosives parent compounds and TNT metabolites in biota exposed to these compounds at other sites. As in the case of the data on biotransfer of organics, the number of empirical BSAF values is extremely limited.

5 Conclusions and Recommendations for Research

Conclusions

This study provides data that can be used to predict exposure-based effects of TNT in aged soil on two plant and two worm species. These data can be used for defining criteria or reference values for environmental management and conducting specific risk assessments.

1. Plant and worm species were more tolerant of TNT during short-term exposure than during long-term exposure.

2. Seeds were less sensitive to TNT than plants.

3. An EC20 of 2.4 mg kg^{-1} soil DW and EC50 of 2.7–7.2 mg TNT kg^{-1} were found for plants.

4. An EC20 of 1.2 mg kg^{-1} and EC50 of 2.2–3.6 mg TNT kg^{-1} were found for worms.

5. All current EC values are lower than most of the published values, which were largely obtained for spiked soils.

6. The following BCF values were found for plant shoots: 1.9 for TNT, 8.5 for RDX, and 14 for HMX.

7. The explosives residues in plant shoots were in the same range as predicted by the BTF equation for TNT metabolites and RDX, but they were lower for HMX.

Recommendations for Research

The toxicity of explosives in aged soils to exposed biota appears to be considerably higher than published on spiked soils. It is recommended to further explore the toxicity of explosives in aged soils to biota and the concomitant bioaccumulation of parent compounds and metabolites, taking the effects of soil-aging processes into consideration.

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Appendix A
Plant, Worm, and Soil Spiking, Explosives Method Detection Limits, and Recoveries

Studies were performed in which the method detection levels were determined for explosives in plants, worm tissues, and soil. Samples were prepared from unexposed reference plant, worm, and soil materials and spiked immediately prior to extraction. Through the sample preparation and clean-up process, concentration factors were introduced that depended on the mass of the substrate tested, the amount of water removed during sample preparation, and the volume of solvents used for clean-up and mobile phase matching (Tables A1 and A2).

The individual effects of 2-h heating to 100 °C and of freeze-drying on the detection levels and recoveries of spiked explosive analytes from spiked samples were quantified also (Tables A1 and A2).

Extraction of Explosives Parent Compounds and Metabolites in Plants, Worms, and Soil

Extraction Procedure 1

Plants were clipped into small pieces and mixed. Subsamples for dry weight analysis were dried in a ventilated oven at 70 °C until constant weight was reached. Subsamples for extraction were homogenized by grinding them in liquid nitrogen. Two-gram FW portions were spiked with 4NT as an internal standard for recovery (50 µL of a 1-mg mL^{-1} solution), heated at 100 °C to remove water, and extracted in 10-mL acetonitrile by an 18-h sonication in a water-cooled bath at 15°C. The extracts were freed from particles by centrifugation for 10 min at 2,000 g. HPLC analysis of the extracts was performed after cutting the supernatants 1:1 with Millipore-filtered RO water, recentrifugation, and clean-up

Table A1
Determination of Method Detection Limits for Each Analyte Spiked into the Reference Soil, Plant Tissue, and Worm Tissue Using the Following Three Procedures: Sonication With Heating, Freeze-Drying, and Sonication Without Heating. Values[1] Are in mg kg^{-1} DW

Analyte	*Lolium perenne*		*Eisenia fetida*		Soil	
	Spike L[2]	MDL[2]	Spike L[2]	MDL[2]	Spike L[2]	MDL[2]
Procedure 1: Sonication with heating[3]						
TNT	217	0.081±0.003	217	BD	NA	NA
2ADNT	217	0.103±0.002	217	BD	NA	NA
4ADNT	217	0.161±0.014	217	BD	NA	NA
4NT	217	0.314±0.017	217	BD	NA	NA
RDX	217	0.142±0.020	217	BD	NA	NA
MNX	217	NA	217	BD	NA	NA
TNX	217	NA	217	BD	NA	NA
HMX	217	0.110±0.015	217	BD	NA	NA
Procedure 2: Freeze-drying[3]						
TNT	617	1.136±0.165	329	1.174±0.107	NA	NA
2ADNT	617	2.016±0.062	329	1.750±0.075	NA	NA
4ADNT	617	2.009±0.291	329	1.773±0.151	NA	NA
RDX	617	3.230±0.239	329	2.176±0.088	NA	NA
MNX	617	5.454±0.922	329	3.194±0.060	NA	NA
TNX	617	0.621±0.060	329	0.456±0.021	NA	NA
HMX	617	7.540±0.615	329	1.645±0.126	NA	NA
Procedure 3: Sonication without heating[3]						
TNT	NA	NA	NA	NA	26	1.684±0.032
2ADNT	NA	NA	NA	NA	26	3.043±0.141
4ADNT	NA	NA	NA	NA	26	1.225±0.408
RDX	NA	NA	NA	NA	26	3.122±0.270
MNX	NA	NA	NA	NA	26	12.353±0.238
TNX	NA	NA	NA	NA	26	1.195±0.112
HMX	NA	NA	NA	NA	26	1.913±0.047

[1] Values represent means of three replicates.
[2] Spike levels given for reference (mg kg^{-1} DW).
[3] TNT, 2ADNT, 4ADNT, and 4NT analyzed using a C18 column; RDX, MNX, TNX, and HMX using a CN column.
Abbreviations: BD, below detection; NA, not applicable.

over a 0.5-g Florisil column. The extraction procedure for worms was similar to that for plants, with one exception: clean-up included filtration over a 0.45-µm polytetrafluoroethylene (PTFE) disk. Fresh subsamples, extracted as described above but without heating at 100°C, were analyzed also. The recoveries of spiked explosives in these plant extracts were lower than in the previously heated extracts, and in these worm extracts far lower than in the previously heated plant extracts. For this reason, alternative extraction procedures were pursued.

Table A2
Recoveries for Explosives Analytes (percent of spike)[1]

Analyte	*Lolium perenne*		*Eisenia fetida*		Soil	
	Spike L[2]	Recovery	Spike L[2]	Recovery	Spike L[2]	Recovery
Procedure 1: Sonication with heating[3]						
TNT	217	46.4±6.7	217	BD	NA	NA
2ADNT	217	45.3±4.7	217	BD	NA	NA
4ADNT	217	41.9±9.2	217	BD	NA	NA
4NT	217	25.9±1.0	217	BD	NA	NA
RDX	217	87.2±18.1	217	BD	NA	NA
MNX	217	NA	217	BD	NA	NA
TNX	217	NA	217	BD	NA	NA
HMX	217	85.0±6.3	217	BD	NA	NA
Procedure 2: Freeze-drying[3]						
TNT	617	19.3±2.8	329	80.1±7.3	NA	NA
2ADNT	617	32.4±2.2	329	88.2±3.8	NA	NA
4ADNT	617	19.3±2.8	329	59.9±5.1	NA	NA
RDX	617	71.7±5.3	329	83.9±3.4	NA	NA
MNX	617	68.6±11.6	329	100.4±2.3	NA	NA
TNX	617	18.6±1.8	329	49.5±2.3	NA	NA
HMX	617	23.3±1.9	329	60.1±4.6	NA	NA
Procedure 3: Sonication without heating[3]						
TNT	NA	NA	NA	NA	26	10.4±0.2
2ADNT	NA	NA	NA	NA	26	15.1±0.7
4ADNT	NA	NA	NA	NA	26	4.8±1.6
RDX	NA	NA	NA	NA	26	99.5±8.6
P;MNX	NA	NA	NA	NA	26	72.6±1.4
TNX	NA	NA	NA	NA	26	17.0±1.6
HMX	NA	NA	NA	NA	26	12.3±0.3

[1] Values represent means of three replicates.
[2] Spike levels given for reference (mg kg^{-1} DW).
[3] TNT, 2ADNT, 4ADNT, and 4NT analyzed using a C18 column; RDX, MNX, TNX, and HMX using a CN column.
Abbreviations: BD, below detection; NA, not applicable.

Extraction Procedure 2

Plants were freeze-dried. Aliquots equivalent to 0.7 g FW were extracted as follows. The freeze-dried material was transferred into bead-beater vials, amended with 0.8-mL acetonitrile, pulverized by two successive cycles of 1-min bead-beating at room temperature (22–24 °C), and sonicated for 2 h at 15 °C. The extracts were freed from particles by centrifugation for 10 min at 2,000 g. HPLC analysis of the extracts was performed after cutting the supernatants 1:1 with Millipore-filtered RO water, recentrifugation, and clean-up over a 0.5-g Florisil column (EPA Method 8330; USEPA 1992). The extraction procedure for worms was similar to that for plants, with two exceptions: sonication lasted 1 h, and clean up included filtration over a 0.45-µm polytetrafluoroethylene (PTFE) disk.

Extraction Procedure 3

Two grams FW of soil was extracted in 10-mL acetonitrile by 18-h sonication at 15 °C, cleaned up over Florisil, and concentrated 10×.

Explosives Analyses

All extracts were analyzed for the following explosives parent and degradation compounds: TNT, 2ADNT, 4ADNT, RDX, the mono-nitroso and trinitroso-derivatives of RDX (MNX and TNX, respectively), and HMX. TNT, 2ADNT, and 4ADNT were analyzed on the C18 column. These compounds separated well, but MNX and TNX coeluted with ADNTs and DANTs, and HMX sometimes fused with the injection peak on this column. RDX, MNX, TNX, and HMX were analyzed on the CN column. These compounds separated well, but TNT, 2ADNT, and 4ADNT coeluted together on this column.

Detection Limits

Detection limits for several target compounds in plants, worms, and soil varied with compound (Table A1) and were lower in freshly extracted than in freeze-dried plants. Detection limits were expressed as method detection level (MDL) in mg kg^{-1} DW as follows:

- In freshly ground and heated plant tissues: TNT 0.081, 2ADNT 0.103, 4ADNT 0.161, 4NT 0.314, RDX 0.142 mg kg^{-1} DW.
- In freeze-dried plant tissues: TNT 1.136, 2ADNT 2.016, 4ADNT 2.009, RDX 3.230, MNX 5.454, TNX 0.621, HMX 7.540 mg kg^{-1} DW.
- The grinding and heating procedure for fresh worm tissues failed to generate detectable levels of the spiked explosives standards.
- In freeze-dried worm tissues: TNT 1.174, 2ADNT 1.750, 4ADNT 1.773, RDX 2.176, MNX 3.194, TNX 0.456, HMX 1.645 mg kg^{-1} DW.
- In freshly ground soil: TNT 1.684, 2ADNT 3.043, 4ADNT 1.225, RDX 3.122, MNX 12.353, TNX 1.195, HMX 1.913 mg kg^{-1} DW.

Recoveries

Significant loss of the spiked analytes can be attributed to freeze-drying of the spiked plant material prior to extraction. However, these losses appear to be highly reproducible within specific tissue samples used. The losses may vary greatly depending on the matrix, from 30-percent recovery for MNX to 81-percent recovery for TNT in plant tissues (Table A2).

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14. ABSTRACT

The objectives of this study were to provide data that can be used to predict exposure-based effects of TNT in aged soil on four endpoint organisms representing two trophic levels. These data can be used for defining criteria or reference values for environmental management and for conducting specific risk assessment.

Dose-response experiments formed the basis for evaluating the toxic effects and transfer of contaminants from soil into two trophic levels, taking bioavailability-modifying soil characteristics into account. Short-term exposure tests were conducted to explore the acute toxicity for the test organisms of TNT-spiked artificial soils and of the aged TNT-contaminated soil to be included in the subsequent long-term exposure tests. In these tests, plants were exposed for 10 days, and seed germination was determined. Worms were exposed for 14 days, and survival was recorded. Long-term exposure tests were conducted to evaluate chronic, sublethal toxicity and transfer of aged soil-based explosives, with TNT as the main contaminant. In these tests, plants were exposed for 55 days in the greenhouse, biomass was determined, and residues of explosives parent compounds and TNT metabolites were analyzed using HPLC techniques. Worms were exposed for 28 days (*Eisenia fetida*) and 42 days (*Enchytraeus crypticus*) in the laboratory, biomass and number were determined, and tissues were analyzed for explosives compounds.

(Continued)

15. Subject Terms		
Aged soil	Eisenia	Lolium
Biotransfer	Enchytraeus	Medicago
	Explosives	Toxicity

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14. ABSTRACT (Concluded).

Both plant test species, *Lolium perenne* and *Medicago sativa*, tolerated TNT concentrations up to 171 mg kg^{-1} dry weight (DW) during short-term exposure. In the longer term the plants were less tolerant of TNT, but *L. perenne* was more tolerant than *M. sativa*. An effective concentration causing a 20 percent decrease in plant biomass (EC20) of 2.4 and EC50 of 7.2 mg TNT kg^{-1} soil DW was derived for *L. perenne* from linear regression. An EC50 of ≤2.7 mg TNT kg^{-1} was found for *M. sativa*, based on the observation that these plants died at TNT concentrations >5.4 mg kg^{-1}. TNT metabolites (2ADNT, 4ADNT), RDX, and HMX were recovered in *L. perenne* shoots and roots. Only the TNT metabolite concentrations in shoots increased significantly with soil TNT concentration.

Among the worm test species, *E. fetida* tolerated TNT concentrations up to 100 mg kg^{-1} during short-term exposure. Fifty percent of these *E. crypticus* individuals died at a TNT concentration as low as 10 mg TNT kg^{-1}, and all died at higher concentrations.

In the longer term the worms were less tolerant of TNT, but *E. fetida* was more tolerant than *E. crypticus*. An EC20 of 1.2 and EC50 of 3.6 mg TNT kg^{-1} soil DW was derived for *E. fetida* from linear regression. An EC50 of ≤2.15 mg TNT kg^{-1} was found for *E. crypticus*, based on the observation that these worms died at a TNT concentration of 4.3 mg kg^{-1}. No explosives parent compounds or metabolites were recovered in the worms. Because only the effects of soil-TNT concentration on the biomass of *L. perenne* and *E. fetida* were significant, the toxicity of the soil was attributed mainly to the contamination by TNT. However, the other explosives identified in the soil mixtures prior to the tests may have contributed also. Clay amendment did not significantly affect the plant and worm responses.